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PROPERTIES OF THE NUCLEIC ACID ISOLATED FROM HVJ

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SUMMARY High molecular weight RNA was isolated from purified HVJ particles and HVJ infected PS cell culture using the SDS-phenol method. Analysis by sucrose density gradient centrifugation indicated that high molecular weight RNA of HVJ sedimented faster than PS cellular ribosomal RNA. The sedimentation coefficient of this RNA was estimated as 42 *S* by analytical centrifugation.

The base composition, susceptibility to ribonuclease and buoyant density of 1.68 of RNA of HVJ suggest that this RNA has a single strand.

INTRODUCTION

The growth of HVJ has been investigated by an autoradiographical technique in our laboratory, and it was recognized that the RNA of HVJ was synthesized in the presence of an appropriate concentration of actinomycin D (HOSOKAWA *et al.* 1963). The fine structure of HVJ virus particles has also been studied by electronmicroscopy by HOSAKA *et al.* (1966). However nothing is known about the size and structure of the RNA in the HVJ virus. Therefore it was of interest to investigate the physical and chemical properties of the RNA of HVJ in relation to previous findings. In our laboratory high molecular, viral RNA was isolated from purified HVJ virus particles and virus infected PS cell cultures by the SDS-phenol method, and its properties were analyzed as described in the present report.

MATERIALS AND METHODS

1. *Virus*

HVJ (Z strain) was used in all the experiments described here. The allantoic cavity of 10 day old embryonated eggs were inoculated with 10^2–10^3 EID_{50} of HVJ virus. After incubation at 35.5°C for 72 hours the eggs were chilled and the chorioallantoic fluid was harvested. The cell debris was removed by centrifugation at 7,000 rpm for 10 minutes. To obtain ^{32}P-labelled virus 1 mc/egg of ^{32}P-phosphate and virus were inoculated simultaneously. To obtain ^{3}H-labelled virus 300 μc/egg of tritiated uridine and virus were injected together.

2. *Virus purification*

The infected chorioallantoic fluid was centrifuged at 22,000 rpm for 35 minutes in a Hitachi 40P centrifuge fitted an RP 30 rotor. The virus pellets obtained were resuspended in PBS (0.14 M NaCl,

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0.02 M phosphate buffer, pH 7.4) and then treated with deoxyribonuclease and ribonuclease at 37°C for 20 minutes (in each case the enzyme concentration was 10 μg/ml in PBS containing 10^{-3} M $MgCl_2$). The virus was then washed three times by differential centrifugation and the final pellets were resuspended in Tris-NaCl buffer (10^{-2} M Tris-HCl buffer, pH 7.4, 0.1 M NaCl). ^{32}P-labelled virus was placed on the top of a 4.4 ml linear sucrose gradient column (15–45 per cent sucrose in 0.01 M Tris-HCl buffer, pH 7.4, 0.1 M NaCl and 0.001 M EDTA) and centrifuged in a Hitachi 40P, RPS 40 rotor, at 25,000 rpm for 90 minutes.

3. *Isolation of viral RNA*

One tenth volume of 1.0 M acetate buffer (pH 5.2) was added to 10^5 HAU/ml of virus suspension (in Tris-NaCl buffer, pH 7.4) and then 20 per cent sodium dodecyl sulfate (SDS) was added to give a 1 per cent solution. The virus suspension was gently shaken and it became somewhat clear. Then an equal volume of water saturated phenol (previously distilled) was added, and the suspension was shaken vigorously. The phenol treatment was repeated three times. The RNA in the aqueous phase separated by centrifugation at 10,000 rpm for 10 minutes, was precipitated by addition of one tenth volume of 20 per cent sodium acetate and two volumes of cold ethanol. The precipitated RNA was resuspended in 0.01 M acetate buffer (pH 5.2) and used as the RNA sample.

Radioactive RNA was also isolated by the same procedure, except that PS cellular RNA or HVJ viral RNA was added as carrier before the RNA was precipitated with ethanol.

4. *Isolation of RNA from infected cell culture*

Porcine kidney stable (PS) cells which were grown in YLE medium containing 10 per cent calf serum in 5 per cent CO_2-air mixture were used. The cell monolayer in the logarithmic phase of growth was infected with virus. Then the cells were incubated in LE (PO_4 free) medium containing 2 per cent calf serum and 0.1 μg/ml of actinomycin C_1 (Serva Entwicklungslabor, Germany). After 3.5 hours the cells were exposed to 300 μC/ml of ^{32}P in the same medium for 90 minutes. The labelled cells were washed three times with PBS, scrubbed and resuspended in TM buffer (0.01 M Tris-HCl buffer, pH 7.4, 0.005 M $MgCl_2$). Deoxyribonuclease (Worthington Biochemical Corp. Freehold. N.J.) was added to make a concentration of 10 μg/ml. The cell suspension was frozen and thawed alternately three times. Then 0.1 volume of 1.0 M acetate buffer and 0.5 per cent final concentration of SDS were added with gentle shaking. This procedure was the same as that described for extraction of viral RNA. RNA from uninfected cells was extracted in the same way.

5. *Sucrose density gradient centrifugation*

RNA samples of 0.2 ml were layered on the top of a 4.4 ml linear sucrose gradient column (9–30 per cent sucrose in 0.01 M Tris-HCl buffer, pH 7.4 containing 0.05 M KCl) and centrifuged at 35,000 rpm for 210 minutes at 15°C in a Hitachi 40P centrifuge, fitted with an RPS 40 rotor. Fractions of 6 drops of the gradient were collected by puncturing the bottom of the tube. The optical density of each fraction was estimated at 260 mμ and then RNA was precipitated with 10 per cent trichloroacetic acid (TCA) and the radioactivity estimated.

6. *Cs_2SO_4 equilbrium density gradient centrifugation*

Cs_2SO_4 was added to the RNA sample resuspended in 0.1 M NaCl, 0.01 M Tris-HCl buffer (pH 7.4), at an initial density (ρ) of 1.64. Then the mixture was centrifuged at 33,000 rpm for 20 hours. Samples were fractionated in the same way as described above and precipitated with carrier RNA by 10 per cent TCA. The precipitate was collected on a filter. The radioactivity of the tritium was counted in a Tricarb liquid scintilation counter.

7. *Analysis of base composition of RNA of HVJ*

The RNA of HVJ precipitated by ethanol was hydrolyzed with 0.3 N KOH at 37°C for 16 hours and neutralized with perchloric acid (PCA). The resulting hydrolysate was analyzed by column chromatography on Dowex-1 to determine its base composition following Bautz and Hall's method.

RESULTS

1. *Purification of virus grown in embryonated eggs*

HVJ virus which had been purified by differential centrifugation was placed on the top of a 15–45 per cent linear sucrose density gradient column and centrifuged at 25,000 rpm for 90 minutes. As shown in Fig. 1, the

virus titer was recognized as a single band in the gradient after centrifugation and the peak of RNAs (recognized by ^{32}P activity) extracted by the phenol method from each fraction also coincided with the peak of virus. This shows that there is no contamination of the purified HVJ virus sample with cellular ribosomal RNA. On the basis of the above results we considered this purified virus suitable for use in subsequent experiments.

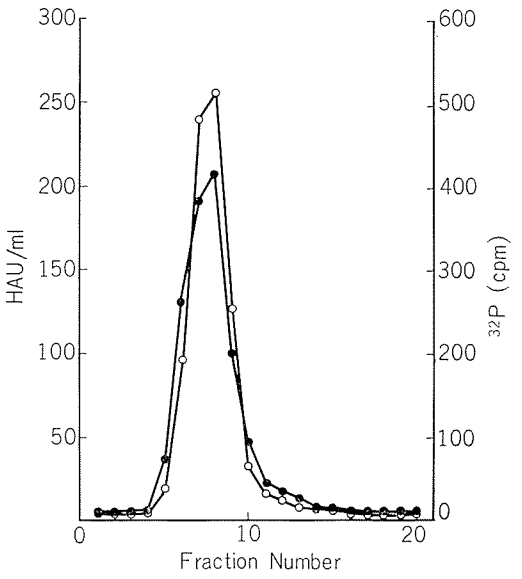

FIGURE 1 Distribution of HA and ^{32}P-labelled RNA of Purified Virus in a Sucrose Density Gradient Column
—○— Hemagglutinating activity
—●— Radioactivity of ^{32}P-labelled RNA

2. *Sucrose density gradient centrifugation of RNA of HVJ*

The properties of virus RNA were analyzed by sucrose density gradient centrifugation. RNA was extracted from ^{32}P-labelled HVJ virus by the SDS-phenol method. As a marker of optical density, PS cellular RNA was added to the viral RNA sample, and the RNA mixture was centrifuged in a sucrose density gradient column. As shown in Fig. 2, the main peak of viral RNA (located by ^{32}P-activity) sedimented much faster than the cellular ribosomal RNA. A small peak of ^{32}P activity was also seen in the upper fractions. Similar small peaks of viral RNA have been reported to be present on density gradient centrifugation of the RNAs of NDV and RSV (DUESBERG, P. H. and ROBINSON, W. S. 1965, and ROBINSON, W. S. *et al.* 1965). From these results we calculated the S_{20w} value as 41.6 using Nomura's formula (NOMURA, M. *et al.* 1960) using cellular ribosomal RNA*s* with 28 *S* and 18 *S* as markers.

In several experiments the amount of small components increased when the virus sample had been stored for a long time after harvesting. The possibility of aggregation of viral RNA with cellular RNA added as carrier was excluded the same peak was seen when viral RNA was analyzed without carrier RNA in the presence of EDTA. These peaks disappeared after treatment of the sample with 0.1 μg/ml pancreatic ribonuclease (Worthington Biochemical Corp. Freehold, N.J.) at 37°C for 20 minutes. From this result the RNA of HVJ seems to be almost completely acid soluble and may be single stranded.

3. *Sucrose density gradient centrifugation of the virus specific RNA from HVJ infected PS cells*

It has been reported that HVJ can be grown in PS cells with a EID_{50}/HA ratio of 6 (HOSAKA, Y. 1962), and that actinomycin D (1.0 μg/ml: product of Merck, Sharp and Dohme) did not affect virus production (HOSOKAWA *et al.* 1963). We investigated the virus specific RNA synthesized in the early stage of infection of PS cells in the presence of actinomycin C_1 (0.1 μg/ml), which specifically inhibits cellular RNA synthesis, but not viral RNA synthesis of HVJ. The RNA extracted from infected cells was analyzed by sucrose density gradient centrifugation under the same conditions as the viral RNA from virus particles. The results are presented in Fig. 3, a and b. This figure shows the virus specific RNA present in HVJ infected PS cells treated with actinomycin C_1. The

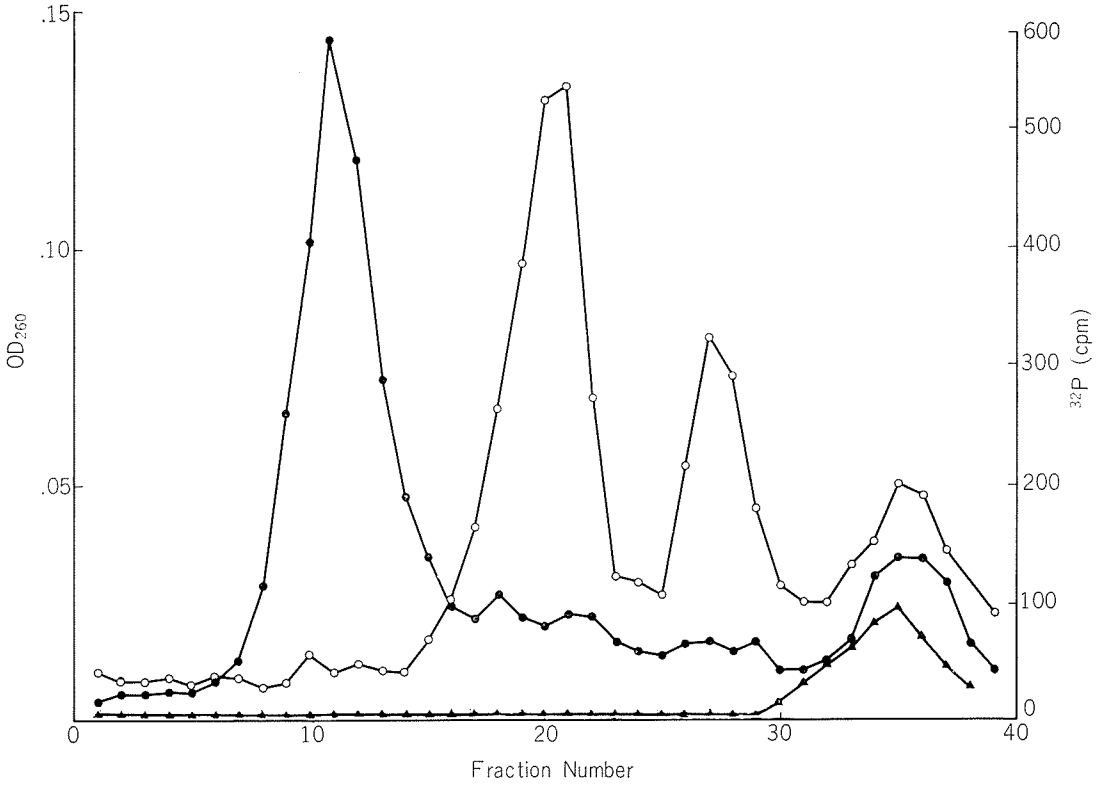

FIGURE 2 Sucrose Density Gradient Centrifugation of ^{32}P-labelled RNA of HVJ
—●— Radioactivity of ^{32}P-labelled HVJ-RNA
—▲— Radioactivity of ^{32}P-labelled HVJ-RNA (treated with RNase)
—○— Optical density of PS cell RNA

peak corresponding to virus specific RNA was not detected in an uninfected cell culture. From above results it was recognized that the high molecular weight viral RNA seen in mature virus particles was also present in infected PS cells 5 hours after infection.

4. *Sedimentation on analytical ultracentrifugation*

RNA was isolated from purified HVJ virus particles as described in the methods section and fractionated by sucrose density gradient centrifugation. The main component obtained was regarded as intact RNA. The fractionated RNA was precipitated with cold ethanol and then resuspended in Tris-HCl buffer containing 0.1M NaCl to give 1.0 OD_{260}/ml. RNA samples were examined in a Hitachi UCA-1

FIGURE 3 Sucrose Density Gradient Centrifugation of RNA Extracted from Infected or Uninfected Cells in the Presence of Actinomycin C_1
(a) HVJ infected cells
(b) uninfected cells
—○— Optical density of PS cell RNA
—●— Radioactivity of ^{32}P-labelled RNA

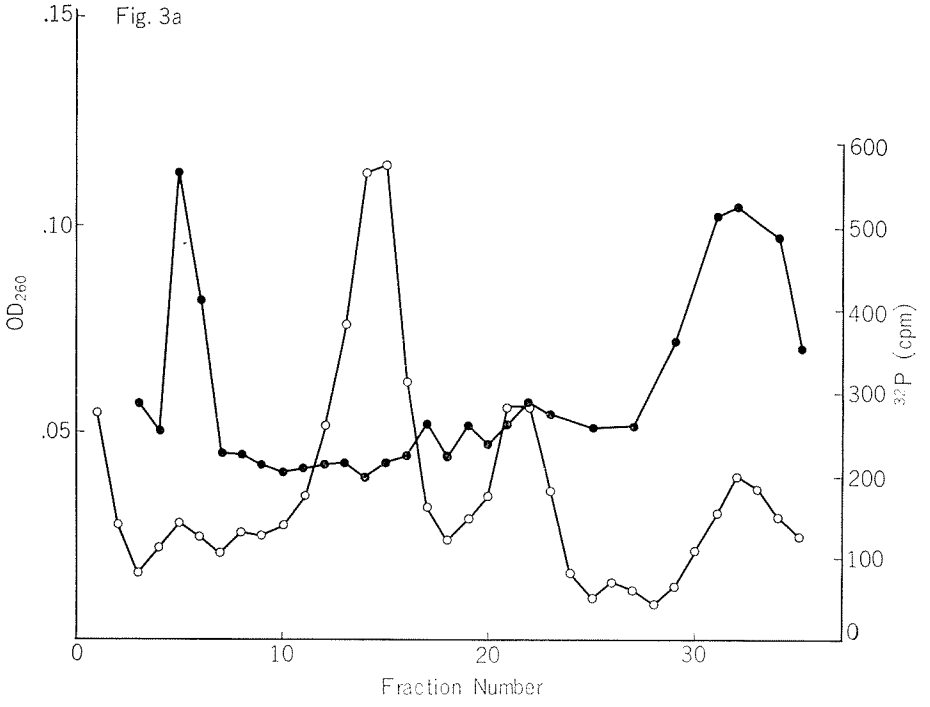
Fig. 3a
.15
.10
.05
OD260
600
500
400
300
200
100
0
32P (cpm)
0
10
20
30
Fraction Number

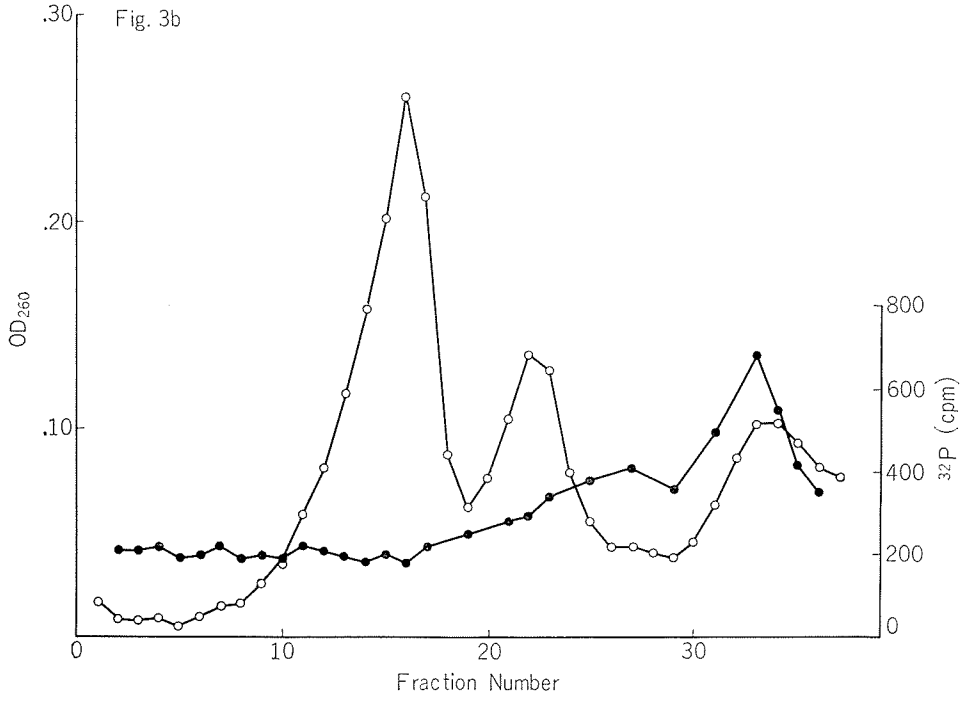
Fig. 3b
.30
.20
.10
OD260
800
600
400
200
0
32P (cpm)
0
10
20
30
Fraction Number

analytical ultracentrifuge fitted with UV optics. Centrifugation were carried out at 40,370 rpm at 20°C. Densitometer tracings of the sedimentation of the RNA of HVJ are shown in Fig. 4. The result shows that most of the RNA was sedimented with a sharp boundary. The sedimentation constant calculated using the sharp portion of the boundary was 42.1 S_{20w} for the RNA of HVJ. This value coincides well with that which was obtained in the sucrose density gradient centrifugation experiment.

5. *Buoyant density of HVJ-RNA in cesium sulfate*

To determine the buoyant density of HVJ-RNA, tritiated viral RNA was resuspended in 0.01M Tris-HCl (pH 7.4) containing 0.1M NaCl, and Cs_2SO_4 was added to give an initial density of 1.64 (ρ). The pattern developed on centrifugation is shown in Fig. 5. The result indicates that the tritium activity appeared as a single peak, with a density of 1.68. This value was similar to those of the single stranded RNAs of poliovirus and Japanese encephalitis virus (JEV) (BISHOP *et al.* 1965; IGARASHI *et al.* 1964) and larger than that of double stranded RNA of Poliovirus in the replicating form. This supports the idea that the HVJ-RNA extracted is single stranded.

6. *Analysis of the base composition of HVJ-RNA*

Extraction of HVJ-RNA with SDS-phenol and precipitation by ethanol were reported, to remove the associated protein of virus RNA before it was hydrolyzed by 0.3N KOH. The precipitated RNA was dried with ethanol-ether (3 : 1) and resolved in a little KOH solution. The hydrolyzed RNA was neutral-

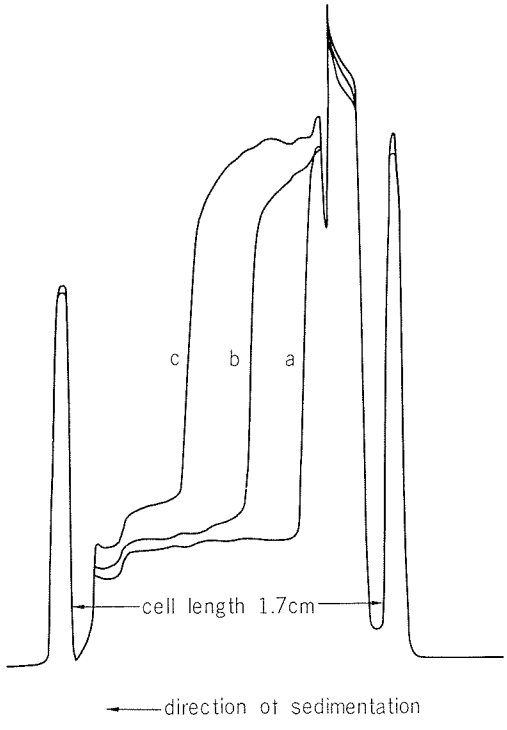

FIGURE 4 Ultraviolet Patterns taken during Analytical Ultracentrifugation of RNA of HVJ
(a) boundary after 1 minute centrifugation
(b) boundary after 10 minutes centrifugation
(c) boundary after 20 minutes centrifugation

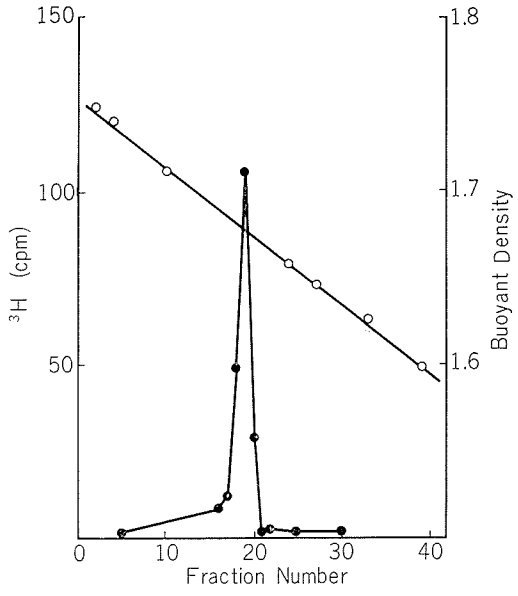

FIGURE 5 Equilibrium Density Gradient Centrifugation of ^{3}H-labelled RNA in Cs_2SO_4
—○— Density of Cs_2SO_4 solution
—●— Radioactivity of ^{3}H-labelled HVJ-RNA

ized with concentrated PCA, and then its base composition was determined by column chromatography on Dowex-1 (Cl form). The results showed that the amount of uridilic acid in HVJ-RNA was more than those of the other three nucleotides (Table 1). This base composition resembles those of NDV and Influenza virus (DUESBERG and ROBINSON 1965; AGRAWAL and BRUENING 1966).

TABLE 1 *Base Composition of RNA of HVJ*

	mol % of nucleotide			
	CMP	AMP	UMP	GMP
exp. 1	25.1	24.4	28.0	22.5
exp. 2	24.0	23.8	31.2	21.0
exp. 3	23.7	24.5	30.9	20.9
average	24.3	24.2	30.0	21.5

DISCUSSION

Several investigations have been reported on the isolation of RNA from myxoviruses. SOKOL *et al.* (1963) reported that the RNA of influenza virus was 10 *S*. However, 38 *S* RNA was isolated from influenza virus by AGRAWAL *et al.* (1966). DUESBERG *et al.* (1965) reported on the isolation of intact RNA from NDV (47 *S*). RNAs of high molecular weight have also been isolated from RSV and mouse leukemia virus (ROBINSON *et al.* 1965; DUESBERG and ROBINSON 1966). It was thought from their size that these RNAs were present as one molecule per one particle. These facts indicate that RNAs of myxoviruses are generally much larger molecules than those of poliovirus and tobacco mosaic viruses. From our results on the RNA isolated from HVJ, the sedimentation coefficient was calculated as 42 *S*. This value is comparable with those obtained for RNAs from other myxoviruses.

In our experiments HVJ-RNA was ribonuclease sensitive and its base composition was not complementary. The buoyant density of HVJ-RNA in cesium sulfate is similar to those of the RNAs of poliovirus and JEV. In experiments on HVJ infection of PS cells, virus specific RNA synthesis was recognized in the presence of actinomycin C_1. These results show that HVJ-RNA is single stranded. Therefore the molecular weight of this RNA may be calculated as that of single stranded RNA from Spirin's formula

$$\text{Molecular Weight} = 1550 \times (S_{20w})^{2.1}$$

which gives a value of 4.0×10^6.

The molecular weight estimated from the length of nucleocapsid, which was measured by HOSAKA (1966) by electronmicroscopy, was $4.5-5 \times 10^6$ molecular weight per one nucleocapsid. This value agrees with the value calculated from the sedimentation coefficient (42.1 *S*), so that this RNA is regarded as intact viral RNA.

AGRAWAL *et al.* (1966) reported on the degradation of influenza virus RNA. Thus intact RNA with a sedimentation coefficient of 38 *S* could be degraded into two specific components (19 *S* and 7*S*) separable by sucrose density gradient centrifugation. They reported that the degradation of viral RNA was stimulated by heating. We also observed the degradation of HVJ-RNA, particularly after storage of the virus preparation. However, it was uncertain whether the degraded RNA was specific.

The similarity in the base composition of the RNAs of HVJ and NDV and influenza virus may represent a common feature of myxoviruses.

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